Kinin B_1 receptor is involved in mechanical nociception in a fibromyalgia-like model in mice

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Reviewer A:

• In general, concise and well written paper, though I have some comments for clarifying the details of the experiment and analysis / presentation of the data.

Reply: **We would like to thank you for the positive comments about our paper. We take this opportunity to thank your relevant questions and suggestions, which will certainly improve our paper's quality.**

• How was the sample size calculated – for measuring neurotransmitters 3-4 per group, for behavioural assays 8-12 per group?

Reply: **The experimental N was based on previous literature data and pilot experiments (Nagakura, Y et al 2009 and Klein, CP et al 2014). Based on our previous publication (Klein, CP et al 2014) and limited budget, we decided to select 3-4 animals per group (vehicle+vehicle and reserpine+vehicle) to assayed the levels of neurotransmitters.**

• Please show the exact number of mice for each group in the figure legend or behind respective symbols on figures (counting the individual cases shown on figures is not that easy).

Reply: **Thank you so much for this suggestion. We included the number of each group in figure 2. Also, we added this information in figure legend. Please see figure 2.**

• The strain name – please correct C57/BL6 to C57BL/6 and complete according to international nomenclature

Reply: **Thank you so much for pointed out our mistyping. We fixed this mistake in the manuscript.**

• Was the age of 8 weeks in the beginning of study? How long the animals were allowed to acclimatize in your facility before the experiment?

Reply: **The C57BL/6 mice were acclimatized in our facility for two weeks before the experiments. The KOB1 mice pups were born in our facility. These information were added in the manuscript.**

• *Was the body weight similar between WT and KO mice?*
Reply: **No significant difference in the body weight was observed before and during the experiment in both strains.**

• *Using inbred strain as control for knockout line may cause problems – could you please comment on that, did you consider it as a limitation, was the background of KO mice verified (as originating from different facility compared to WT mice, i.e. possible environmental and genetic differences between the groups)?*
Reply: **Based on our previous experience and publication (Viana, AF et al; 2010 and Maciel, IS et al; 2020-preprint), no significant difference was observed in both strains (body weight, behavior and pharmacological effect). Unfortunately, we did not assay the genetic differences between the groups. However, we are very confident with behaviour and pharmacological answer in both strains**

• *Circadian cycle – when were the lights on / off?*
Replay: **The lights were on 7 am and off 7pm. We added this information is the manuscript.**

• *Please describe briefly housing conditions (IVC / open cages, enrichment)?*
Replay: **Animals were housed in groups of four or five per cage (30 x 20 x 13cm) in microisolator cages equipped with inlet/outlet air filters and the cages were filled with autoclaved wood chip bedding. This information was added to the manuscript.**

• *The last sentence in Methods – I assume 96 mice required more than one day for testing, please describe if blocking was applied, and if the experimenters were blinded for the treatments also during von Frey test?*
Replay: **Each experimental session included at least 4-6 animals of each treatment group and the experiment was repeated 2-3 times. The investigators were blinded during the behavior tests. This information was added to the manuscript.**

• *How long approximately the von Frey test took for individual animals? Important to know for calculating the time between pharmacological treatments and forced swim test. Were the mice moved to home cage (grouped) or into a separate transfer cage for 30 min interval between PWT and FST? Please add to the description of measuring PWT that it's known also as von Frey test (this name is used later in the figure legend).*
Reply: **The mice were individually acclimated for one hour in elevated clear plexiglass boxes with a wire mesh floor. After the first 30 min of acclimatization, the mice were removed from the box to pharmacological treatment (i.p.) and immediately returned to plexiglass box. The von Frey test begin after thirty minutes of injection, in which the mice completed one hour of acclimatization in the plexiglass box. In the end of von Frey test (PWT), the mice remained in the plexiglass box for 30 minutes and were carefully transferred to adjacent room to perform the FST. The FST test was performed one hour after the pharmacological treatment. The mice without i.p. injection (figure 2B and 2D) were exposed to the same protocol without the manipulation during the plexiglass box acclimatization. This information was added to the manuscript.**

• As Dixon Table is mentioned in the methods, would be fair to refer also to original paper (Dixon, 1980)?
Reply: **This reference was mentioned in the manuscript (reference 18).**

• Please remove = between F and parentheses showing d.f.
Reply: **Thank you for that, we removed the parentheses**

• I wonder about P values - all reported values (except one) equal 0.0001 – is this correct?
Reply: W**e revised the statistical data analysis and corrected information (p values) were added in the manuscript.**

• For analysis of behavioural assays, it remains unclear which interactions exactly are reported and what ANOVA's were calculated in specific cases (one-way, two-way, or repeated measures)? Please specify the factors and levels for ANOVA and report the main effects followed by interactions.
Reply: **We revised our statistical analysis. Two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons post hoc test was used to investigate the differences in the PWT (factors = time and treatment-group). In the case of interaction between the factors, a one-way ANOVA followed by Bonferroni multiple comparisons post hoc test was performed in test session (supplemental file). In the FST, one-way ANOVA followed by Bonferroni multiple comparisons post hoc test was used. The statistic values were described in the results and discussion session.**

• Symbol # is not explained in the legend for Figure 2.
Reply: **The Symbol # means a significantly differences (P < 0.05) from the reserpine+vehicle or reserpine+wt group. These information were added in the manuscript.**

• On Figure 1, I assume the 100% level is calculated from the values of control (vehicle-treated) mice. However, KOB1 PFC-Vehicle seem to have well below 100%?
Reply: **Thank you for noticing this error. We identify a typing mistake and we fixed the data. We replaced the figure on the manuscript.**

Reviewer B

Comments and questions from reviewer B:

The paper focuses on validating a fibromyalgia mouse model and the role of the kinin receptor in mediating behavioral responses. Overall, the paper is well-written and the flow of the text is good. However, the statistical methods need major revision.
Reply: We would like to thank you for the positive comments about our paper. We take this opportunity to thank your relevant questions and suggestions in our statistical data analysis, which will certainly improve our paper's quality.

Figure 1 B: What is exactly analyzed with the 2-way ANOVA?
What are the factors in this ANOVA? Based on the df (5, 33), the brain region/treatment were not included as separate factors, although this is expressed as the main finding of this experiment.
If the main question is the effect of reserpine on serotonin levels in different brain regions, then the two independent variables should be treatment (vehicle and reserpine) and brain regions (PFC,

Hippocampus, Spinal cord) either analyzed per group (wt or KOB1) or the genotype also added as a factor.

"a significant reduction of serotonin (F = (5, 33) = 85.54, p = 0.0001; N = 3-4, figure 1B) and dopamine (F = (5, 32) = 29.67, p = 0.0001; N = 3-4, figure 1C) levels into each structure evaluated" From this, it is not clear what this significant effect here is. The ANOVA results should be more clearly indicated. The interaction/main effects should be written out. In the figure legend it is indicated that this was followed by post-hoc testing, but this is not written in the results section. Post-hoc comparisons should be included (what was compared with what) together with the statistics of these comparisons (t-value with n and p-value).

Reply: Thank you for these relevant questions. Our main goal was to assess the effect of reserpine in the serotonin/dopamine levels in the PFC, HPC and spinal cord. The differences in the dopamine and serotonin levels between vehicle and reserpine groups (in each sample region) were analysed by Student's t test. To facilitate readers' visualization, we created one figure with 2 graphs (Figure 1A and 1B). The statistic values were described in the results and discussion session.

Also, in text "Both wild type and KOB1 mice showed a similar reduction in serotonin and dopamine levels induced by reserpine". Based on what analysis? Was the genotype included as a factor (see previous comment)?

Reply: In this paragraph, we would like to say that both strains showed a similar profile on serotonin and dopamine reduction. Also, the KOB1 mice were not protected against the reserpine effect on amine levels in the PFC, HPC and spinal cord. The statistical analysis was revised and non-significant difference were observed in serotonin levels (WT versus KOB1, one-way ANOVA = F (5, 18) = 6.235) in the PFC (t = 1.351, p = 0.5802), HPC (t = 0.878, p = 0.99) and spinal cord (t = 2.107, p = 0.1481). On the other hand, a significant difference was observed in dopamine levels (WT versus KOB1, one-way ANOVA = F (5, 18) = 17.92) in PFC (t = 5.250, p = 0.0002), HPC (t = 2.640, = 0.499) and spinal cord (t = 4.288, p = 0.0013). We rewrote the sentence with the new statistical data analysis.

Here the part that was rewritten: "Both strains showed a similar profile in the decrease of serotonin and dopamine after reserpine injection. A similar decrease in serotonin level was observed in both strains (WT versus KOB1, one-way ANOVA = F (5, 18) = 6.235) in the PFC (t = 1.351, p = 0.5802), HPC (t = 0.878, p = 0.99) and spinal cord (t = 2.107, p = 0.1481, figure 1B). On the other hand, a significant difference was observed in dopamine levels (WT versus KOB1, one-way ANOVA = F (5, 18) = 17.92) in PFC (t = 5.250, p = 0.0002), HPC (t = 2.640, = 0.499) and spinal cord (t = 4.288, p = 0.0013, figure 1C)."

Figure 2 A-B:

2A: The ANOVA analysis is not clear. Is this a repeated two-ANOVA? Why and what were the factors here? Shouldn't the analysis be repeated one-way ANOVA in this experiment?

Reply: Based on our data (different groups and two-moment of the assay), we used two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post hoc test in the PWT (factors = time and treatment-group). In the case of interaction between the factors, a one-way ANOVA followed by Bonferroni's multiple comparison post hoc test was performed in the test session (supplement file). Very similar results were found when we did one-way (ANOVA) in the test session (FIGURE A: (F (3, 36) = 29.08; pregabalin: t = 6.119, p = 0.0001 and R-715: t = 8.170, p = 0.0001; N = 8-12; supplement file) or FIGURE 2B (F (3, 37) = 20.35.48, p = 0.0001; N =

10-11; supplement table). In the baseline, no significant differences between the groups were found. The details of post-hoc comparisons were added in the manuscript.

"….the acute treatment with pregabalin (30 mg/kg; i.p.; 30 min. before behavioral test) or with B1R antagonist R-715 (0.5 mg/kg, i.p; 30 min. before behavioral test), significantly inhibited the mechanical allodynia induced by reserpine (interaction = F (3, 36) = 25.40, p = 0.0001, N = 8-12; figure 2A)"

Significant interaction effect between what? Also, details of post-hoc comparisons missing.

Reply: Significant interaction in the test session between the reserpine+vehicle group versus reserpine+pregabalin or reserpine+R-715 group. The details of post-hoc comparisons were added in the manuscript.

2B: If this is repeated two-way ANOVA, the factors should be clearly indicated.

"…B1R receptor knockout mouse (KOB1R) showed protection against the fibromyalgia related mechanical hypersensitivity (interaction = F (3, 37) = 8.49, p = 0.0002,N = 10-11; figure 2B)"

Significant interaction effect between what? Also, details of post-hoc comparisons missing.

The df 3,37 suggest that the genotype, treatment and time were not included as separate factors, instead the analysis included time as a factor but genotype/treatment were not separated?

Reply: We considered as a factor: 1 (baseline and test) and 2 (experimental group-treatment), the significant interaction in the test session between the reserpine+wt group versus reserpine+KOB1 group. The details of post-hoc comparisons were added in the manuscript.

2C: "….only the treatment with R-715 significantly reduced the immobility time in FST (F (3, 36) = 37.42 p = 0.0001, N = 8-12; figure 2C)". If this is a one-way ANOVA, it should be clearly indicated. Details of post-hoc comparisons missing.

2D: "….increase the immobility time in the FST (interaction = F (3, 37) = 10.10, p = 0.0001, N = 10-11; figure 2D".

Reply: The details of post-hoc comparisons were added in the manuscript.